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Comparison of fecal culture and F57 real-time PCR for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in swiss cattle herds with a history of paratuberculosis

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Contents

1 Abstract

Fecal samples from a total of 1339 cattle (855 animals from 12 dairy herds and 484 animals from 11 suckling cow herds, all herds with a history of paratuberculosis) were investigated by culture and by real-time PCR for *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Pools of three samples each were decontaminated by means of a NaOH/oxalic acid method, prior to inoculation of Loewenstein-Jensen (LJ) and Herrold's Egg Yolk agar (HEYA) with mycobactin. Cultures were incubated at 37°C for 16 weeks and monitored for MAP growth at biweekly intervals. A qualitative real-time PCR targeting the F57 insertion element of MAP was used to detect MAP in fecal pools and to identify MAP grown in culture. By culture, MAP was detected in 62 of 445 fecal pools (13.9%); whereas the F57 real time PCR detected MAP in 9 of 445 pools (2.0%). All 186 samples of the 62 culture-positive pools were reanalyzed individually: MAP was grown from 59 individual samples (31.7%). By PCR, MAP was detected in 12 samples (6.5%). All PCR positive samples tested positive by culture and none of culture-negative samples tested positive by PCR. In conclusion, culture is more sensitive to detect MAP in feces from clinically unaffected cattle than F57 real-time PCR. The main reason for the striking differences between culture and PCR is that the amount of fecal extract subjected to culture is significantly higher than the corresponding aliquot that could be analyzed by a single PCR run.

Zusammenfassung

Es wurden Kotproben von 1339 Rindern (855 Tiere aus 12 Milchkuhbetrieben, 484 Tiere aus 11 Mutterkuhbetrieben, alle Betriebe mit mindestens einem bestätigten Fall von Paratuberkulose) mittels Kultur und Real-time PCR auf *Mycobacterium avium* subspecies *paratuberculosis* (MAP) untersucht. Jeweils 3 Proben wurden gepoolt und mit der NaOH/Oxalsäure Methode dekontaminiert, bevor sie auf Löwenstein-Jensen (LJ) und auf Herrold's Egg Yolk Agar (HEYA) verimpft wurden. Die Kulturen wurden bei 37°C für 16 Wochen inkubiert und alle zwei Wochen auf das Wachstum von MAP geprüft. Parallel wurden die Kotproben in einer qualitativen Real-time PCR untersucht, die das F57 Insertions-Element von MAP detektiert. Diese PCR wurde auch verwendet, um MAP-verdächtige Bakterien aus der Kultur zu identifizieren. In der Kultur wurde MAP in 62 der 445 Pools (13.9%) nachgewiesen; in der PCR erwiesen sich 9 Pools (2.0%) als MAP-positiv. Alle 186 Proben der 62 kulturell positiven Pools wurden einzeln nachuntersucht: MAP war kulturell in 59 Einzelproben (31.7%) und mit der PCR in 12 Proben (6.5%) nachweisbar. Alle PCR-positiven Proben waren auch in der Kultur positiv und keine der kulturell negativen Proben ergab ein positives PCR-Resultat. Um MAP im Kot von klinisch unauffälligen Rindern nachzuweisen, ist die Kultur im Vergleich zur F57 Real-time PCR erheblich sensitiver. Der Grund dafür ist, dass in der Kultur im Vergleich zur PCR ein um den Faktor 10^3 grösseres Inokulum untersucht werden kann.

2 Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the etiological agent of paratuberculosis (Johne's disease), an incurable chronic granulomatous enteropathy primarily affecting wild and domestic ruminants. MAP-infected cattle may have implications for public health because the agent is putatively linked to a human chronic granulomatous ileitis (Crohn's disease) (Bull et al. 2003, Chacon et al. 2004, Greenstein et al. 2004). In the dairy industry, paratuberculosis has been globally recognized as an important cause of morbidity and hence, of economic loss (Ott et al. 1999, Nielsen et al. 2009, Timms et al. 2011).
In European countries, the prevalence of paratuberculosis amongst cattle is approximately 20% (Nielsen et al. 2009). In Switzerland, the rate is considerably lower, i.e. at 10%, on average (Glanemann et al. 2004, Bogli-Stuber et al. 2005, Nielsen et al. 2009). However, the significance of MAP as a pathogen in the Swiss cattle population is hard to estimate. Only the very small number of an average of 15 cases of clinical bovine paratuberculosis is reported to the Swiss Federal Veterinary Office (FVO) each year, out of the overall cattle population of approximately 1.6 million animals.

Bearing this in mind, systematic analyses of both prevalence and clinical relevance of MAP in Swiss dairy cattle are indicated. A number of diagnostic tests, e.g. bacterial culture, gene detection assays, and serological assays are available (Collins et al. 2006). Paratuberculosis is diagnosed mainly through direct confirmation of MAP in feces, by either bacterial culture, or by PCR targeting specific MAP gene sequences. However, studies comparing culture and PCR for the detection of MAP in bovine feces have often disclosed unclear or contradictory results: In fact, a total of 35 publications on this particular comparison can be found (papers accounted for until May 2013). Indeed, only 9 papers (25.7%) indicate the stage of infection/health of the animals tested or of their herds, respectively. Such data is, however, essential for reevaluating studies comparing MAP detection by PCR and culture, as irregular fecal shedding of usually few MAP cells occurs during the pre-clinical stage. By contrast, high numbers of MAP are shed during the late clinical stage (Timms et al. 2011). Thus, the sensitivity of both culture and PCR depend on the stage of infection. Our

analysis of literature shows that, except for one study (Logar et al. 2012) for the category of low MAP shedders, culture is more sensitive than PCR. In the category of high shedders, however, PCR sensitivity is not superior to culture (table 1). Compared to this broad-based evidence, the above-mentioned study (Logar et al. 2012) results in completely opposite outcomes: Here, 89% of 141 clinically healthy cattle from a herd with a known history of paratuberculois were identified as MAP-shedders by a qualitative real-time PCR; whereas only 19% were identified as MAP-shedders by culture. To verify this striking discrepancy, we compared fecal samples of 1339 clinically healthy cattle from herds with a history of paratuberculosis by means of culture and PCR testing. The systematic comparison of both methods, using samples from a large stock of naturally infected animals, should prove which method provides a higher likelihood for the direct confirmation of MAP in fecal samples obtained from apparently healthy cattle from herds with a history of paratuberculosis.

3 Materials and methods

3.1 Herds and Animals

The present study involved 1339 cattle from 23 herds, i.e. 855 animals (including 7 bulls) from 12 dairy herds and 484 animals (including 22 bulls) from 11 suckling cow herds. Herd sizes ranged from between 11 to 130 animals. The average age in the dairy herds was 4.2 years (minimum: 10 months, maximum: 13 years) and 4.8 years in the suckling cow herds (minimum: 11 months, maximum: 17 years). During the last 5 years, each herd had at lea st one animal which was reported to the FVO notifiable diseases registration office with confirmed paratuberculosis. 3 cows showed clinical signs indicative of paratuberculosis, i.e. 2 cows aged 3.5 and 5.5 years, respectively but from different suckling cow herds (cantons of Valais and Fribourg) as well as one cow 3 years of age from a dairy herd (canton of Fribourg). The geographical spread of the 23 herds among the Swiss cantons is illustrated in figure 1.

3.2 Sampling

Fecal samples were collected by veterinarians from the Clinic for Ruminants, Vetsuisse Faculty University of Berne, over a period of 5 months, i.e. from February to July, 2011. Samples were collected directly from the rectum with a glove without lubricant, subsequently chilled and sent to the Institute of Veterinary Bacteriology, Vetsuisse Faculty University of Zurich. The samples were stored at -20°C until testing (for a maximum period of 3.3 months). At the time of sampling, all animals were clinically healthy, except for three cows presenting clinical signs indicative of paratuberculosis (i.e. 2 cows aged 3.5 and 5.5 years, respectively but from different suckling cow herds (cantons of Valais and Fribourg) as well as one cow 3 years of age from a dairy herd (canton of Fribourg).

3.3 Pooling and Decontamination Procedure

Fecal samples were assayed in batches, according to their sampling date. Individual samples were pooled solely within the same herd. Thus, pools consisted mainly of three individual samples, but two or four samples were also possible. Pooling was performed by weighing 2.0 g of feces from each of the 3 cows into a sterile 50 ml polycarbonate screw capped tube (Becton Dickinson, Basel, Switzerland). 8.0 ml of sterile water and glass beads (4 mm Ø, Faust, Schaffhausen, Switzerland) were added to improve homogenization by vigorous vortexing. Subsequently, two 3.0 ml aliquots of the homogenate were subjected to the decontamination procedure according to Beerwerth (1967). Briefly, each 3 ml aliquot was homogenized in 40 ml of 4.0% NaOH (Roth, Karlsruhe, Germany) with glass beads by repeated agitation on a vortexer, shaken for a further 8 min on a horizontal shaker and then allowed to stand for 2 min in order for gross particles to settle. The supernatants were then pooled. Upon centrifugation (3000 x g, 15 min, 20°C) the pellet was suspended again, in 20 ml of 5% oxalic acid by thorough repeated agitation on a vortexer and was then shaken for 15 min on a horizontal shaker. The suspension was centrifuged as described above. The pellet was then suspended again, in 6 ml of sterile saline (0.15 M NaCl) and used as inoculum.

3.4 Culture

Loewenstein-Jensen (LJ) medium containing 2.0 mg/l mycobactin J (Enclit, Oelzschau, Germany) and Herrold's Egg Yolk Agar (HEYA) supplemented with 50 µg/ml vancomycin, 50 µg/ml nalidixic acid, and 50 µg/ml amphotericin B (Becton Dickinson, Basel, Switzerland) were used as culture media. 200 µl aliquots of the inoculum were transferred to each of two slants of LJ and HEYA, respectively. The inoculated tubes were left to stand for 1 h at ambient temperature so that the agar surface was horizontal, thus permitting maximum absorption of the inoculum onto the agar surface (Kalis et al. 1999). Inoculated tubes were incubated at 37°C for 16 weeks and monitored for bacterial growth at biweekly intervals. MAP was preliminarily diagnosed on the basis of colony morphology (small, smooth to slightly rough, opaque to whitish colonies). All presumptive positive colonies were picked up with sterile swabs and transferred into 3 ml Middlebrook 7H9 broth, supplemented with ADC-enrichment (Becton Dickinson, Basel, Switzerland), and 2.0 mg/l Mycobactin J (Synbiotics Europe, Lyon, France). Broth cultures were incubated for at least one week at 37°C and microscopically tested for acid-fast staining bacteria, following Ziehl-Neelsen staining (Ziehl 1882, Neelsen 1885). All cultures with mycobacterial growth confirmed by microscopy were subjected to a F57-PCR specific for MAP. Fecal samples of all pools testing positive for MAP were reanalyzed individually by culture and PCR to identify the MAP shedders.

3.5 Preparation of genomic DNA from fecal samples

A combined mechanical lysis step with the High Pure template preparation kit (Roche Diagnostics GmbH, Penzberg, Germany) was used to isolate genomic DNA from bovine fecal samples. Approximately 500 to 700 mg of a fecal sample was suspended again in 2.0 ml of phosphate buffered saline (Roche Diagnostics GmbH, Penzberg, Germany). 100 µl of this solution was then suspended once more in 130 µl lysis buffer supplied with the kit; 20 µl Proteinase K were then added. The mixtures were first incubated at 65°C for 30 min, then at 95°C for 10 min. Following this, they were transferred on to the lysing matrix in the MagNA lyser tubes. A mechanical step consisting of 1 min at 6500 rpm in the MagNA lyser instrument was repeated three times. After the last step, 120 µl were applied to the MagNA Pure System (Roche) for

DNA-purification. From the resulting 90 µl eluate, a 2 µl aliquot was used as a template for PCR analysis.

3.6 Real-Time PCR

Real-time PCR was used to co-amplify and detect a 254-bp target region in the MAP F57 sequence and a 257-bp IC template (Bosshard et al. 2006). Reactions were performed in the LightCycler 2.0 instrument (Roche Molecular Diagnostics) in a total reaction volume of 20 µl in glass capillary tubes. The reaction mixture contained 1 x LightCyclerFaststart DNA master plus hybridization probes mix (Roche Molecular Diagnostics), 1.00 nM of each primer (MAP f57p1, MAP f57p2), 200 nM of each LC probe (MAP f57-3'Fluo, MAP f57-5'LC Red 640, puC19-5'LC Red 705), 2 µl DNA template, and 20 copies of IC template. The amplification consisted of an initial pre-incubation step at 95°C for 10 min to activate the DNA polymerase, followed by 45 cycles of 95°C for 10 s, 56°C for 20 s, and 72°C for 18 s. The fluorescence signals corresponding to the F57 sequence target and the IC template amplification were monitored during the 56°C annealing step in the LC Red 640 nm and LC Red 705 detection channels of the LightCycler 2.0 instrument, respectively.

4 Results

By culture, MAP was detected in 62 of 445 fecal pools (13.9%). In detail, 48 isolates (77.4%) were grown only on HEYM, 2 (3.2%) only on LJ, and 12 (19.4%) on HEYM and LJ, respectively. MAP was grown within 9 to 11 weeks of incubation. Of the 1780 cultures (445 pools, 4 agar slants per pool), 16 (0.9%) were heavily contaminated by constituents of the bovine fecal flora and were thus excluded. However, investigation of the pools in question could be finalized, since there was only one medium tube per pool that could not be evaluated. Using F57 real time PCR, MAP was directly detected in 9 of the 445 pools (2.0%).

To identify the MAP shedders, all 186 samples of the 62 culture-positive pools were reanalyzed individually by culture and by PCR. MAP was isolated from 59 of the 186 individual samples (31.7%) representing 50 of the 62 MAP-positive pools (80.6%). In

detail, we identified 43 pools with 1 positive sample, 5 pools with 2 positive samples, and 2 pools with 3 positive samples (Σ 59 MAP isolates). By PCR, MAP was directly detected in 12 samples (6.5%) from 11 pools (10 pools with 1 positive sample; 1 pool with 2 positive samples). All PCR-positive samples also tested positive by culture. None of the culture-negative samples tested positive by PCR.

Overall, MAP was detected in 10 of 13 dairy cow herds (76.9%), and in 8 of 10 suckling cow herds (80.0%). Results of the MAP tests and relevant herd data are summarized in table 2.

Additionally, we evaluated a total of 17 animals showing clinical signs indicative of paratuberculosis, either when the samples were taken (n=3, re. Materials & Methods) or over an average duration of 7 months after the samples were taken (minimum: 1 month, maximum: 16 months). By culture, MAP was detected in fecal samples of all 17 animals. PCR detected MAP in fecal samples from 12 of the 17 animals (70.6%). Thus, the number of PCR-positive fecal samples from animals suspected of clinical paratuberculosis was significantly higher than with the 169 individual samples from clinically healthy cows ($p<0.0001$, chi^2-test; table 3).

5 Discussion

Within the scope of the present study, we investigated a total of 1339 fecal specimens from apparently healthy cattle from herds with a known history of paratuberculosis. This sample collection provides a valid basis for a comparison of PCR and culture to detect MAP in feces from cattle without clinical signs of paratuberculosis. For this purpose, the cultural assay is significantly more sensitive than the F57 real-time PCR. Cultivation of MAP succeeded in 62 fecal pools (14.7%) as well as in 59 of the 186 (31.6%) individual fecal samples; whereas only 9 fecal pools (2.1%) and 12 individual fecal samples (6.4%) tested positive with real-time PCR. Our finding of a considerably higher analytical sensitivity in culture is being confirmed by the majority of papers published on comparative detection of MAP in bovine feces by culture and PCR in the period between 1992 to 2013 (table 1).

PCR detection methods for MAP are considered less sensitive than bacterial culture (30% sensitivity for PCR versus 60% for culture; Collins et al. 2006, Timms et al. 2011). The reasons for this are as follows: MAP-infected but still apparently healthy cattle (preclinical stage) only shed small amounts of bacteria in their feces in comparison to diseased cattle (Chiodini et al. 1996). The chance of detecting such small amounts of bacteria is much more likely in culture, because a much larger inoculum can be tested than with PCR. According to our protocol governing the processing/decontamination of fecal specimens, the inoculum for culture was equivalent to 336 mg feces. This amount was used to inoculate four agar slants which were subsequently incubated for 16 weeks and examined for growth of MAP at least 8 times (every other week). By comparison, the template for PCR consisted of an extract equivalent to 0.27 mg feces, which was subjected to a single PCR run. Thus, the aliquot subjected to culture was 1244 times higher than the corresponding aliquot analyzed by a single PCR run. Otherwise, when we presume that for a positive PCR reaction, the template needs to contain at least 5 genome equivalents, the limit of detection for our PCR is 3.7×10^3 MAP per gram of feces. In comparison, an inoculum for the *in vitro*-growth of MAP needs to contain 50 viable mycobacteria on average (Krebs 1964, Kralik et al. 2012). In conclusion, for the cultural growth of MAP according to our protocol, a fecal sample must contain no less than 5.9×10^2 MAP per gram of feces, which is approximately one sixth of the MAP numbers required for a positive PCR. The much higher rate of MAP detection in culture than with PCR is also due to the fact that we systematically sub-cultivated all MAP-suspected colonies. An average of two subcultures per sample was applied, taking into consideration all samples (445 pools, 186 individual samples). These considerations explain why culture is more suitable than PCR to identify animals with asymptomatic intestinal MAP infections and low bacterial shedding.

Since our MAP culture is highly labour-intensive and thus probably less feasible for routine diagnostics, the validity of PCR for monitoring bovine paratuberculosis must be estimated. Our results show that upon the onset of paratuberculosis symptoms, animals may be reliably identified as MAP-positive with a single PCR test, because cattle shed larger amounts of bacteria in the clinical state of paratuberculosis. Animals with clinical paratuberculosis in an infected herd are merely the tip of the iceberg (Whitlock et al. 2000). Our tests have proven that the state of infection in

herds (MAP yes/no?) may be clarified quite clearly with clinically affected animals. Applying only PCR is critical when dealing with clinically unaffected herds where, according to our findings, mainly low shedders are expected. In our study, 6 of 18 MAP-positive herds (30.0%) could not be detected by PCR. A recent paper refers to the fact that the systematic optimization of the DNA extraction may allow the sensitivity of the PCR to meet the sensitivity of culture (Leite et al. 2013). However, it remains unaddressed, if the approximately 8 times higher sensitivity of PCR as compared to culture (Logar et al. 2012; Hanifian et al. 2013) is solely on an optimized DNA extraction. In any case, this important technical aspect must be verified within the scope of further systematic studies.

In literature, various approaches to grow MAP from bovine feces are described without any one standard being generally accepted. This applies, in particular, to the decontamination of fecal samples (maximum inactivation of fecal bacterial contaminants whilst preserving viability of MAP). We decided to ensure decontamination by means of NaOH and oxalic acid. This method has been described as being quite effective several times, because very few cultures are lost due to an overgrowth by contaminants from the fecal flora (Beerwerth 1967, Kalis et al. 1999, Kalis et al. 2000, Nielsen et al. 2002, Glanemann et al. 2004, Nielsen et al. 2004, Vansnick et al. 2007, Fernandez-Silva et al. 2011). NaOH is mainly advantageous because an alkaline environment causes less damage to mycobacteria than an acidic environment (Gray et al. 1954, Patterson et al. 1956). Even according to our results, the alkali-coupled oxalic acid-treatment introduced in to the field of mycobacteriological diagnostics 83 years ago (Corper et al. 1930) leads to a considerably higher detection rate of mycobacteria from feces than other decontamination methods: A total of 1279 presumptive MAP-positive bacterial colonies were inoculated into M7H9 bouillon (data not shown). Acid-fast bacteria were grown in 1080 subcultures (84.4%) of which 183 (16.9%) were finally identified as MAP.

In conclusion, the cultural assay is more sensitive for MAP detection in fecal samples from animals without clinical signs compared to a F57 real time PCR. Nevertheless, considering the long cultivation period and the work-intensive procedure involved, its applicability for eradication measures of bovine paratuberculosis remains restricted. The limited performance of PCR is mainly due to the fact that in cultural assays, 1244

times more fecal material is used. Results from this study also highlight the impact of the medium chosen for cultivation, since HEYA revealed a much higher prevalence of MAP than LJ.

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7 Tables

	MAP-status of animals tested	**Sensitivity of methods compared**		
Authors	**Infection status**	**PCR > Culture**	**Culture > PCR**	**PCR = Culture**
Sockett et al. 1992	Low MAP-shedders from herds with a history of Johne's disease		X	
Fang et al. 2002				X
Paolicchi et al. 2003			X	
Taddei et al. 2004			X	
Wells et al. 2006			X	
Eamens et al. 2007			X	
Clark et al. 2008			X	
Pozzato et al. 2011			X	
Logar et al. 2012		X		
Taddei et al. 2004	High MAP-shedders		X	
Wells et al. 2006			X	
Clark et al. 2008				X
Pozzato et al. 2011		X		

Table 1: Detection of MAP in fecal samples from cattle with a history of paratuberculosis: Literature overview.

	Dairy Cows		**Suckling Cows**	
No. of herds	12		11	
No. of animals per herd	65.8		48.4	
No. of pools	283		162	
Bacteriology	Culture	PCR	Culture	PCR
MAP-positive herds	10	4	8	4
Map-positive pools per herd	3.5	0.4	3.4	0.6
MAP-shedders per herd	1-10	1	1-7	1-2
Age of MAP-shedders	4.7		4.8	

Table 2: Herd data and bacteriologic findings in dairy cows and suckling cows.

		Culture		PCR			
Farm	**Date of Sampling**	**Pool**	**Individual Sample**	**Pool**	**Individual Sample**	**Age (Y)**	**Months until Symptoms**
1	15.02.11	+	+	-	-	9	2.1
2	01.03.11	+	+	+	+	5.5	*a*
3	10.03.11	+	+	-	-	4.8	12.2
3	10.03.11	+	+	-	+	6.5	9
3	10.03.11	+	+	-	+	6.5	10
4	13.03.11	+	+	+	+	6.6	8.2
5	29.03.11	+	+	-	-	3.1	8.2
5	29.03.11	+	+	+	+	8	6
6	05.04.11	+	+	+	+	3	*a*
7	19.04.11	+	+	-	-	5.7	8.6
8	10.05.11	+	+	+	+	3.5	*a*
9	16.05.11	+	+	+	+	6.6	1.5
10	23.05.11	+	+	+	+	3.8	4.7
11	25.05.11	+	+	+	+	8.9	7.9
11	25.05.11	+	+	+	+	8.3	7.9
11	25.05.11	+	+	+	+	2.4	8.6
12	30.06.11	+	+	-	-	1.9	15.6

Table 3: Detection of MAP in fecal samples from animals showing symptoms of paratuberculosis at sampling[a)] or during the period after sampling.

8 Figures

Figure 1: Geographical distribution of the 23 cattle herds.

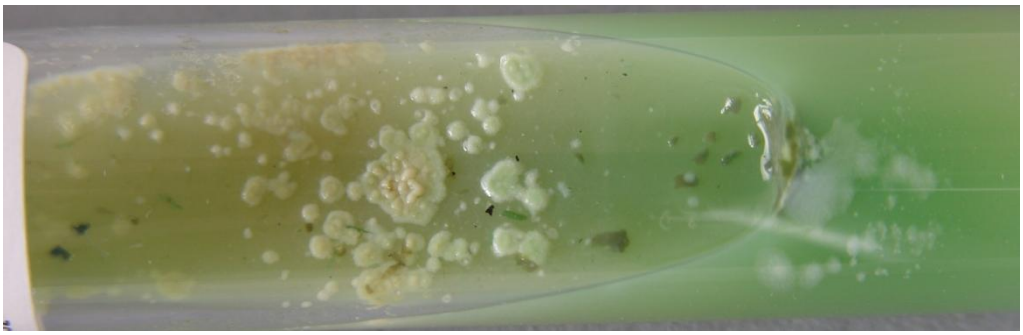

Figure 2: Growth of MAP on HEYM (8 weeks of incubation).

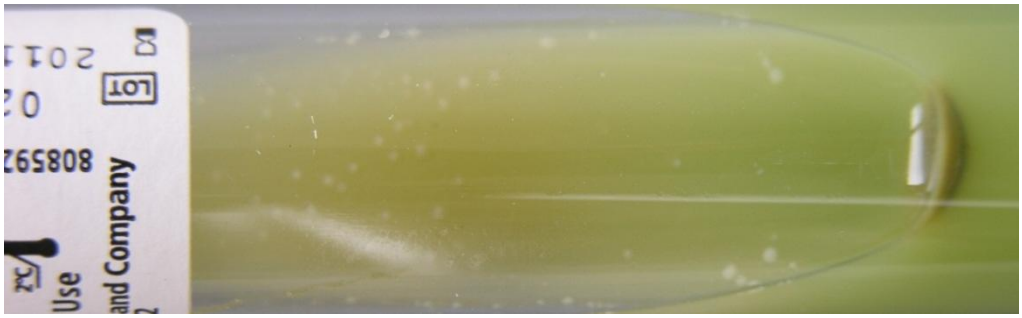

Figure 3: Growth of MAP on HEYM (6 weeks of incubation).

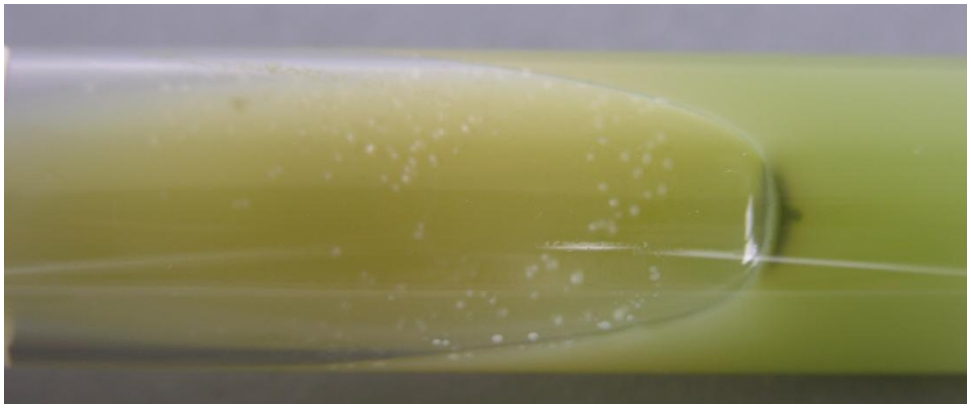

Figure 4: Growth of MAP on HEYM (8 weeks of incubation).

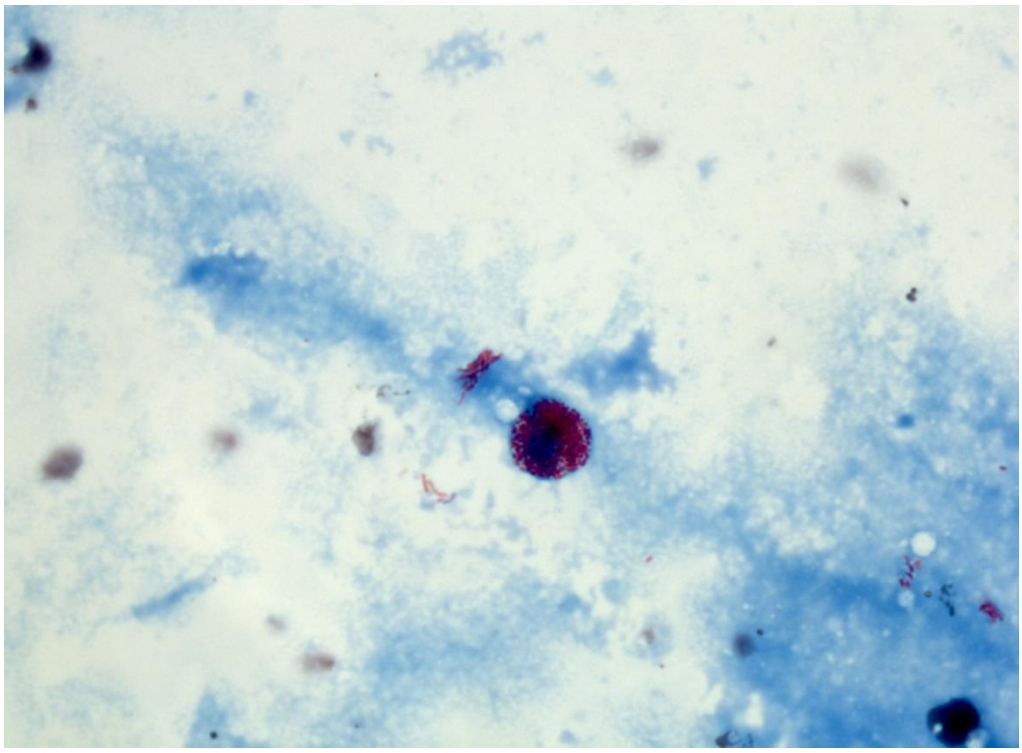

Figure 5: Ziehl-Neelsen staining of MAP in Middlebrook 7H9 broth (low mycobacterial growth, 1 week of incubation).

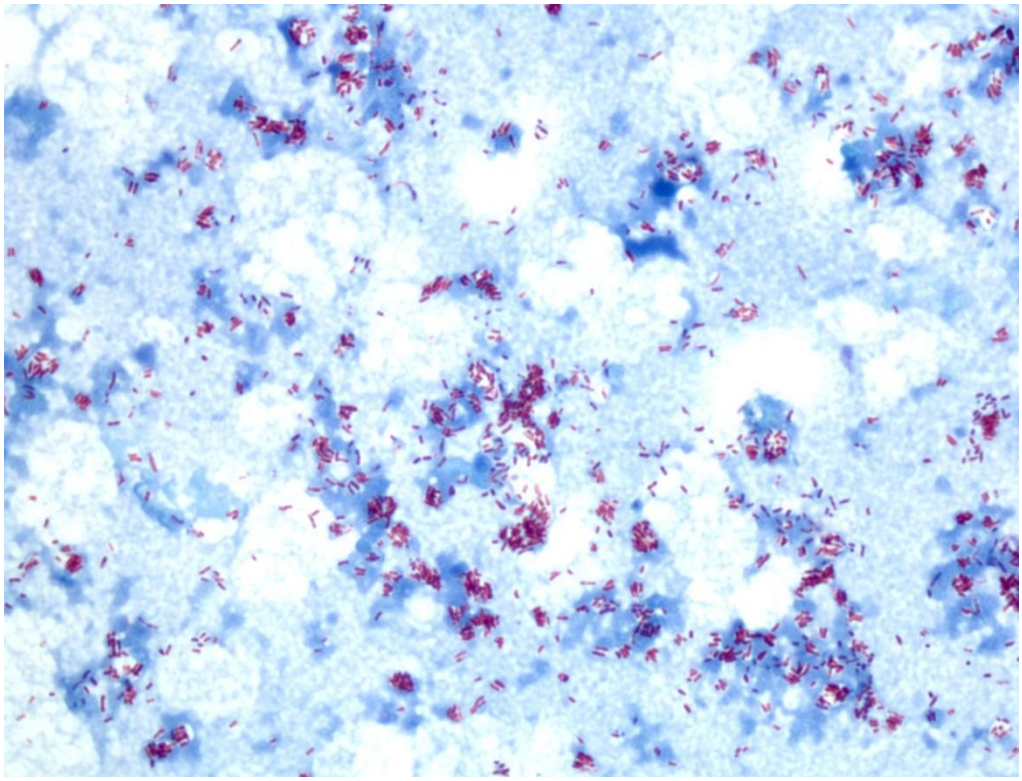

Figure 6: Ziehl-Neelsen staining of MAP in Middlebrook 7H9 broth (moderate mycobacterial growth, 1 week of incubation).

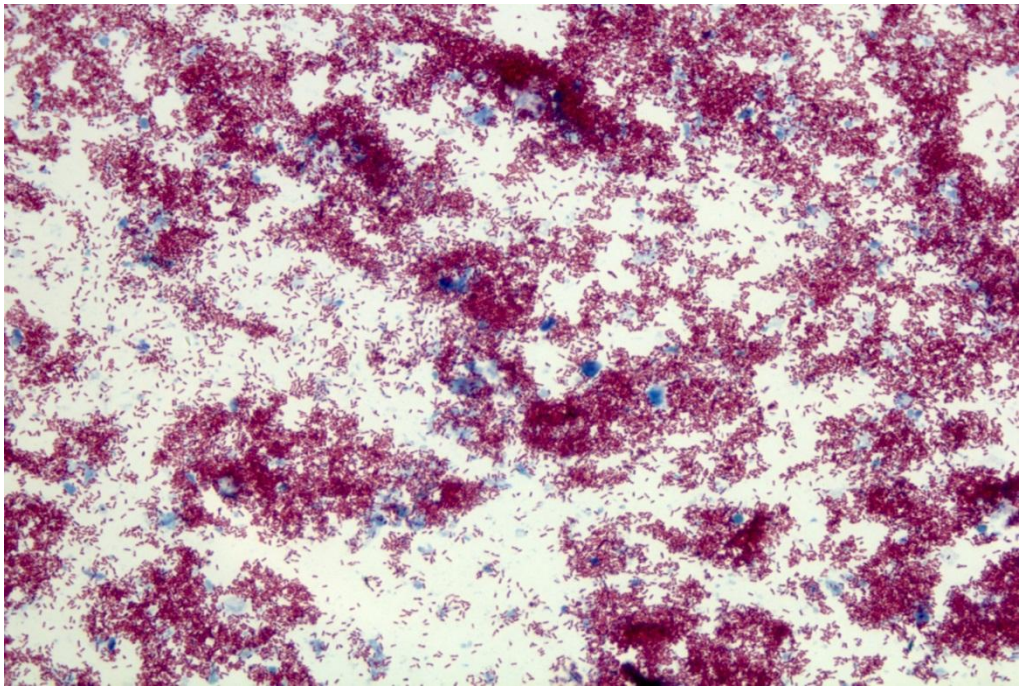

Figure 7: Ziehl-Neelsen staining of MAP in Middlebrook 7H9 broth (heavy mycobacterial growth, 1 week of incubation).

9 Curriculum vitae

Name	Keller Selina Maria
Geburtsdatum	05.07.1984
Geburtsort	Winterthur (ZH)
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8/1998-7/2002	Kantonsschule, Winterthur, Schweiz
8/2002-7/2005	Hochalpines Institut, Ftan, Schweiz Schwerpunktfach: Französisch Ergänzungsfach: Biologie
25.6.2005	Erlangung der Maturität am Hochalpinen Institut Ftan
9/2005-10/2010	Studium der Veterinärmedizin, Universität Zürich, Schweiz
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2/2012-1/2013	Assistentin am Institut für Veterinärbakteriologie, Vetsuisse-Fakultät Universität Zürich, Schweiz
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